

REMARKS

The Claims Are Not Rendered Obvious by Rockwell in View of Ciardiello

Claims 1-6, 9, 11-14, 16-18, 28, 62, and 67-69 stand rejected under 35 U.S.C. 103(a) as being allegedly rendered obvious by Rockwell in view of Ciardiello. Applicants traverse this rejection.

Ciardiello describes administering a combination of a VEGF antisense oligonucleotide and an EGFR antibody to potentially inhibit tumor growth.¹ Rockwell makes no mention of VEGF antisense but rather describes VEGFR antibodies. Applicants submit that one skilled in the art would not be motivated to substitute the VEGF antisense describes by Ciardiello with a VEGFR antibody as described by Rockwell for at least the following reasons.

First, because Ciardiello describes a VEGF antisense oligonucleotide, Ciardiello is directed to inhibiting VEGF expression and eliminating the protein entirely. Therefore, Ciardiello describes preventing VEGF production by tumor cells such that VEGF is not even available to bind to the different VEGF receptors expressed on endothelial cells to thereby activate angiogenesis. Rockwell, on the other hand, describes inhibiting VEGF further downstream, i.e. inhibiting (once produced by tumor cells) the interaction between VEGF with the VEGF receptors expressed on endothelium cells to inhibit angiogenesis (See Figure 4). However, Rockwell indicates that “VEGF mediates its regulatory response on endothelial cells through two high-affinity PRK receptors, mouse flk-1 and its human homologs KDR and a second receptor, flt-1” (See page 321). One skilled in the art would not be motivated to use VEGFR antibodies instead of VEGF antisense because antibodies to both flk-1 and flt-1 may be required to get the same results as a VEGF antisense. In other words, there is no motivation to change a single VEGF knockout approach with an approach that requires antibodies to two different receptors.

Further, as of the filing date of the present application, it was known that neuropilin-1, which binds to VEGF, also mediates angiogenesis via its interaction with VEGF. (See Exhibit A). Therefore, to one skilled in the art considering Rockwell and Ciardiello in view of the general knowledge in the art regarding VEGFR receptors that mediate angiogenesis,

¹ Applicants wish to clarify that there was never an acknowledgment in any previously filed response that Ciardiello showed a synergistic effect upon administering an antisense VEGF oligonucleotide with an EGFR antibody.

binding of VEGF to three different receptors may need to be blocked in order to get the same result as a single VEGF antisense. It is Applicants' position that such a multi-receptor approach undercuts any motivation to replace the VEGF antisense described by Ciardiello with a VEGFR antibody described by Rockwell.

Second, Ciardiello describes using two approaches, VEGF antisense and C225, that both involve reducing the source of VEGF from tumor cells. Namely, VEGF anti-sense inhibits the synthesis of VEGF and C225 inhibits the secretion of VEGF (See Ciardiello page 3742, second column). Ciardiello mentions in the introduction that an anti-VEGF antibody has been generated and that the development of antibodies against the VEGF-specific flk-1/KDR is "a promising approach." Therefore, although Ciardiello recognizes two approaches that target VEGF after VEGF is produced by tumor cells, Ciardiello pursues a dual approach to reducing VEGF from even being produced by tumor cells in the first place. Applicants submit that to one skilled in the art, such a direction of Ciardiello suggests or points to therapeutic approaches that are directed to reducing the source of VEGF. Rockwell, on the other hand, describes antibodies that bind to VEGFR and inhibit binding of VEGF to its receptor and that do not target the production of VEGF.

For at least these reasons, Applicants submit that there is no motivation to combine Rockwell with Ciardiello and therefore these references do not render obvious the present claims. Accordingly, Applicants request withdrawal of this rejection.

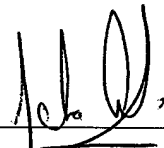
CONCLUSION

It is respectfully submitted that the present application is now in condition for allowance, which action is respectfully requested. The Examiner is invited to contact Applicants' representative to discuss any issue that would expedite allowance of the subject application:

Any fees for extension(s) of time or additional fees required in connection with the filing of this response, are hereby petitioned under 37 C.F.R. § 1.136(a), and the Commissioner is authorized to charge any such required fees or to credit any overpayment to Kenyon & Kenyon's Deposit Account No. 11-0600.

Respectfully submitted,
KENYON & KENYON LLP

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Neuropilin is a mediator of angiogenesis*

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Key words: VEGF, semaphorins, VEGF receptors, plexin, tumor angiogenesis

Abstract

Neuropilin is both a receptor for semaphorins, which are mediators of neuronal guidance, and for VEGF, an angiogenesis factor. While the function of neuropilin in the nervous system has been characterized, its role in angiogenesis is only beginning to be elucidated. This article reviews some of the structural and functional features of neuropilin and presents the experimental evidence showing that it is a mediator of angiogenesis. In particular, we show that neuropilin and its soluble isoforms regulate tumor angiogenesis and subsequent tumor growth.

I. Introduction

Angiogenesis is a prime regulator of tumor growth [1,2]. The induction of tumor angiogenesis is a control point in tumor expansion and in the metastasis of tumor cells to distant sites. There is great interest in characterizing the molecular regulators of angiogenesis, both stimulators and inhibitors. Among the best characterized angiogenesis regulatory systems are vascular endothelial growth factor (VEGF) and VEGF receptors (VEGFRs), fibroblast growth factors (FGF) and FGF receptors, angiopoietin and Tie receptors, and ephrins and Eph receptors (reviewed in [3]). Recently, we purified and cloned neuropilin-1 (NRP1) and neuropilin-2 (NRP2) as novel receptors for VEGF₁₆₅ [4]. NRPs were initially described as receptors for semaphorins, and therefore mediators of neuronal guidance [5–7]. In this review we show evidence that NRPs are also mediators of angiogenesis, for example, in tumors. Thus, there may be molecular cross-talks between the processes of neuronal guidance and angiogenesis.

II. Neuropilin

Neuropilin-1 (NRP1) was first described by Fujisawa and colleagues as a 130 kDa transmembrane glycoprotein expressed on axons in the developing nervous

system [8–12] (reviewed in [13]). It was suggested that NRP1 was involved in neuronal cell guidance, axonal growth and fasciculation in particular neuronal circuits [14]. NRP1 is highly conserved among various vertebrate species, including *Xenopus*, chicken, mouse, rat, and human (reviewed in [13]). Another member of the NRP family, NRP2, shares a 47% homology with NRP1 and is expressed in overlapping, yet distinct populations of neurons in the embryonic nervous system and in many adult tissues [4,5]. Human NRP1 and NRP2 genes span over 112 and 110 kb on chromosomes 10p12 and 2q34, respectively. The genes are comprised of 17 exons. Most of the exons of NRP1 gene are identical in size to the corresponding exons of the NRP2 gene, suggesting gene duplication has occurred (Rossignol and Klagsbrun, submitted).

During embryonic development, NRP1 is expressed in the developing nervous, cardiovascular, and skeletal systems [8,14–17]. NRP1 is also expressed in adult endothelial cells, tumor cells [4], and a variety of tissues including placenta, heart, lung, liver, kidney, and pancreas [4], as well as bone marrow stromal cells [18], osteoblasts [19] (Harper and Klagsbrun, unpublished), and keratinocytes (Bielenberg and Klagsbrun, unpublished). NRP2 has a similar expression profile. Overexpression of NRP1 in mice resulted in sprouting and defasciculation of nerve fibers [16], while targeted disruption of the NRP1 gene in mice resulted in severe abnormalities in the trajectory

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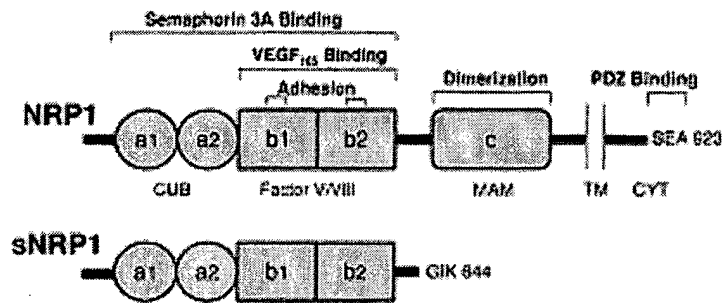


Figure 1. The domain structure and binding sites of neuropilins. (Top) Structure of NRP1. NRP2 has a similar structure. Depicted are the three extracellular domains (a, b, c) and their homologies to various structures (CUB, coagulation factor V/VIII and MAM, respectively), the transmembrane domain (TM), and the cytoplasmic domain (CYT) (adapted from [14]). The c/MAM domain mediates NRP dimerization. The putative binding sites for semaphorin (Sema3A), VEGF₁₆₅, and a PDZ domain containing protein that binds to the three C-terminal amino acids (SEA) of NRP1 and NRP2 are depicted. Adhesion sites in the b/factor V/III domain for cell-cell interactions are also depicted. (Bottom) The structure of soluble NRP1 (sNRP1), an NRP1 isoform that is truncated in the b/c linker region [34]. This protein has 641 amino acids derived from exons, and three additional C-terminal amino acids (GIK) which are intron derived.

of the cranial and spinal efferent fibers that express NRP1 normally [20]. On the other hand, NRP2 deficient mice were viable and reached adulthood but with some adverse effects in the nervous system [21]. The effects on angiogenesis in mice overexpressing NRP1 or deficient in NRP1 and NRP2 will be described below.

NRPs are receptors for class 3 secreted semaphorins [5–7] and for VEGF₁₆₅, VEGF-B, VEGF-E and placental growth factor (PIGF) [4,22–25]. Structural analysis indicates that NRP1 and NRP2 contain extracellular, transmembrane, and cytoplasmic domains as expected for a receptor [11,14] (Figure 1). The extracellular region is composed of three domains, a1/a2, b1/b2, and c. The a1/a2 domain is homologous to complement components C1r and C1s (CUB). The b1/b2 domain is homologous to coagulation factors V and VIII. The central portion of the c domain is designated as MAM (meprin, A5, receptor tyrosine phosphatase μ) and is homologous to these proteins. The cytoplasmic domain is short, about 40 amino acids. There have been attempts to identify proteins that bind to these domains. Although the story is not totally clear at this point, deletion analysis suggests that the a [26,27] and b domains [27,28] are involved in Sema3A binding to NRP1. The b domain is responsible for VEGF₁₆₅ binding [27]. The c domain is involved in NRP1 dimerization [27–30]. NRP1 mediates cell adhesion via heterophilic molecular interactions and parts of the b domain are responsible for these adhesion interactions [12,31]. The 3 C-terminal amino acids of NRP1 (SEA) interact with the PDZ

domain of NRP1-interacting protein [32,33], which is expressed concomitantly with NRP1 [32]. The NRP1-interacting protein has been independently cloned as RGS-GAIP-interacting protein, and has been suggested to participate in clathrin-coated vesicular trafficking. A schematic of putative binding domains of NRP1 is depicted in Figure 1.

Recently, a naturally occurring soluble 90 kDa NRP1 (sNRP1) containing domains a and b of NRP1 was cloned from a human prostate carcinoma cell cDNA library [34] (Figure 1). NRP1 and sNRP1 are synthesized selectively by use of cleavage/polyadenylation (C/P) sites within unprocessed NRP1 or alternatively spliced RNA transcripts. The 2.2 kb sNRP1 mRNA contains a unique, 28 bp intron-derived sequence at the 3' end. Within this sequence, there is a stop codon that results in a 644 amino acids protein that includes 3 unique amino acids at the C terminus [34]. Recently, we have cloned additional soluble NRP1 and sNRP2 species (Rossignol and Klagsbrun, submitted). sNRP1 is a VEGF₁₆₅ antagonist and its effect on tumor vascularization and growth will be described below.

III. Semaphorin/collapsin

Semaphorins/collapsins are a large family of secreted and membrane-associated axon guidance proteins [35,36] (reviewed in [37]). Chick collapsins were the first to be identified [36]. Semaphorins are the corresponding mammalian proteins. There are eight classes of semaphorins characterized by various

structural features [38]. All semaphorins contain a highly conserved 500 amino acids ectodomain known as the semaphorin (sema) domain [38]. Secreted semaphorins are chemorepellants. They are involved in axon steering, fasciculation, branching and synapse formation (reviewed in [37]). Class 3 semaphorins are the only ones that bind to NRPs [5–7]. Semaphorin 3A (Sema3A, formerly SemaIII, collapsin-1) binds to NRP1 with high affinity [7] while Sema3B, Sema3C, and Sema3F bind to both NRP1 and NRP2 with high affinity [7,39]. Sema3A has been fairly well characterized. It is a 95 kDa protein synthesized as an inactive precursor that is activated by furin-dependent proteolytic processing [40]. Sema3A inhibits axon migration and collapses dorsal root ganglion (DRG) neuron growth cones in an NRP-dependent manner [5–7,20,27,39]. Sema3A is also an inducer of neuronal cell apoptosis [41,42]. Mice deficient for Sema3A have defects in the nervous, cardiovascular, and skeletal systems [43].

The mechanisms of semaphorin signaling via NRPs are at present not well understood but some progress has been made. Sema3A binds to NRP a and b domains [26–28]. Dimerization of Sema3A is necessary for its activity [44,45]. The Sema3A signal is mediated by NRP1 homodimers, Sema3F by NRP2 homodimers, and Sema3C by NRP1 or NRP2, either as homodimers or heterodimers [7,29]. Several studies have indicated that NRP alone is not sufficient to convey signals. The short NRP cytoplasmic domain lacks any known signaling motifs. In addition, the transmembrane and cytoplasmic domains of NRP1 are not needed for biological activity [7,11,26,28]. These observations imply that other transmembrane receptors are needed to transduce the Sema3A signal. It is possible that additional molecules may be present as preformed complexes with NRP1 on the cell surface or may be recruited into the NRP1–semaphorin complex after ligand binding [26,28]. One candidate would be plexin 1. Plexins are transmembrane proteins expressed in vertebrates and invertebrates [30,46,47]. Class 3 secreted semaphorins do not bind directly to plexins. Instead, plexin 1 and NRP1 form a complex [30,47]. While plexin 1 alone does not bind sema3A, the NRP1/plexin 1 complex has a higher affinity for Sema3A than does NRP1 alone [30]. Expression of dominant negative plexin 1 in sensory neurons blocks Sema3A-induced growth cone collapse [30]. Thus, physiologic Sema3A receptors may consist of NRP1/plexin complexes. The role of NRP may be to enhance Sema3A binding affinity much the way NRP1 enhances the binding of VEGF₁₆₅

to VEGFR-2 [4]. How plexin 1 transduces a signal is not known. The cytoplasmic domain of plexin 1 is required for Sema3A activity [30,46]. One possibility is that plexin 1-mediated signaling involves tyrosine phosphorylation since the cytoplasmic domain of plexins is associated with tyrosine kinase activity [47]. Based on previous studies analyzing the mechanism of growth cone collapse, cytosolic molecules such as CRMP, Rac1, and cGMP may be the downstream signaling contributors [26,48–50].

IV. VEGF and its receptors

VEGF, a 40–45 kDa homodimeric protein regulates normal embryonic vasculogenesis, physiological angiogenesis, and tumor angiogenesis [3,51–54]. VEGF has been considered primarily to be an EC mitogen and chemotactic factor and a potent angiogenesis factor *in vivo*. However, recently, there is evidence that VEGF stimulates non-EC types, for example, VEGF has been shown to stimulate osteoblast migration and differentiation [19,55], and axonal outgrowth and Schwann cell proliferation and survival [56]. VEGF is expressed by tumor cells and contributes to tumor angiogenesis. Its expression is upregulated by hypoxia and is often elevated in regions of tumor necrosis (reviewed in [57]). Inhibiting VEGF activity in tumors is a novel approach for inhibiting tumor growth [54]. Clinical trials currently underway include the use of humanized anti-VEGF antibodies and of small molecule kinase inhibitors that block the activation of VEGF receptors by targeting VEGFR ATP binding sites.

VEGF monomers exist as 5 different isoforms of 121, 145, 165, 189, and 206 amino acids that are produced by alternative splicing from a single gene containing 8 exons. VEGF₁₂₁ and VEGF₁₆₅ are the most abundant isoforms. Recently, other structurally homologous members of the VEGF family have been identified including PlGF, VEGF-B, VEGF-C, VEGF-D, and VEGF-E (reviewed in [3,51,52,54,58]). Targeted disruption of even one allele of the VEGF gene results in impaired blood vessel formation and growth retardation, suggesting tight dose-dependent regulation of embryonic vessel development by VEGF [59,60].

VEGF activities are mediated by high-affinity receptor tyrosine kinases (RTKs) associated primarily with EC. Two VEGF RTKs have been identified, 180 kDa VEGFR-1 originally known as Flt-1, and

200 kDa VEGFR-2 originally known as KDR/Flk-1 [reviewed in (3,51,52,54,58)]. Targeted disruption of the VEGFR-2 gene in mice resulted in early defects in the differentiation and development of EC [61]. In these mice, yolk sac blood vessels were absent and there were no organized blood vessels at any stage. In targeted disruption of VEGFR-1, normal EC differentiation from precursor cells occurred but EC assembly into blood vessels was impaired [62]. Therefore, VEGFR-2 seems to function earlier than VEGFR-1.

Recently, NRP1, a receptor for semaphorins was identified as a receptor for VEGF as well [4]. NRP1 binds VEGF₁₆₅ but not VEGF₁₂₁. The binding of VEGF₁₆₅ to NRP1 is mediated by the exon 7-encoded region that is absent in VEGF₁₂₁. A fusion protein containing the exon 7 peptide binds to NRP1 and is a VEGF₁₆₅ antagonist [63].

V. Neuropilins mediate angiogenesis

Although initially described as a mediator of neuronal guidance, NRP1 appears also to be a mediator of angiogenesis. It is a receptor for VEGF₁₆₅ [4] and several other members of the VEGF family that are angiogenic [22–24]. It is expressed by EC both *in vitro* and *in vivo* [16,34,64]. NRP2 is also expressed by EC (Bielenberg and Klagsbrun, unpublished). In our initial studies on VEGF/NRP1 interactions, EC lines were generated expressing either NRP1, VEGFR-2 or both NRP1 and VEGFR-2 [4]. It was demonstrated that co-expressing both VEGFR-2 and NRP1 in EC enhanced VEGF₁₆₅ binding to VEGFR-2, and VEGF₁₆₅-mediated EC chemotaxis, compared to EC expressing VEGFR-2 alone. This enhancement did not occur in the presence of VEGF₁₂₁. EC expressing NRP1 alone did not respond to either VEGF isoform, indicating that NRP1 was not a signaling receptor for chemotaxis. These results suggested that in EC, NRP1 acted as a co-receptor for VEGFR-2. VEGF₁₆₅ binds to NRP1 via the VEGF exon 7 encoded peptide [4], and to VEGFR-1 and VEGFR-2 via the exons 3 and 4 encoded peptides, respectively [65]. Thus a possible mechanism for NRP1 enhancement of VEGF binding and activity might involve VEGF₁₆₅ forming a bridge between NRP1 and VEGF RTKs, bringing these receptors in closer proximity (Figure 2). Recently, it was shown that immobilized NRP1 extracellular domain increased the affinity of VEGF₁₆₅ for VEGFR-2 [66]. This study also demonstrated that NRP1 binds directly to Ig-like domains 3 and 4 of VEGFR-1.

A role for NRP1 in angiogenesis has been suggested in animal studies. Overexpression of NRP1 in transgenic mice resulted in embryonic lethality [16]. Besides anomalies in the nervous system such as nerve fiber sprouting at incorrect positions, the cardiovascular and skeletal systems were also affected. In particular, hemorrhaging in the head and neck, and excess capillary and blood vessel formation throughout the embryo were noted. Targeted disruption of the NRP1 gene resulted in embryonic lethality [20,67]. Although there were severe abnormalities in the nervous system, death was actually attributed to anomalies in the cardiovascular system. Various types of vascular defects were observed including impairment of neural angiogenesis, lack of formation of branchial arch-related great vessels and dorsal aorta transposition of great vessels and disorganized and insufficient development of vascular networks in the yolk sac. The vascular regression in NRP1 deficient embryos was in contrast to the overproduction of vessels in the embryo overexpressing NRP1 [16,67]. It was concluded that NRP1 plays a role not only in nerve fiber guidance but in embryonic blood vessel formation as well. On the other hand, many NRP2 knockout mice were viable and no defects in angiogenesis were reported [21].

A basic helix-loop-helix transcription factor, dHAND, is expressed in the developing vascular mesenchyme and in its derivative vascular smooth muscle cells. Targeted deletion of the dHAND gene revealed severe defects in embryonic and yolk sac vascular development [17]. For example, the mesenchymal SMC failed to differentiate into vascular SMC. The mice died from heart failure. NRP1 expression was downregulated in the heart and in the vasculature of dHAND null mutants, suggesting a possible role for dHAND in angiogenesis that might be NRP1- and VEGF-dependent.

VEGF is an EC agonist. In contrast, semaphorins, the other family of NRP ligands, are inhibitors of EC function [68]. Sema3A could bind to EC engineered to express NRP1 but not to parental cells or EC expressing VEGFR-2 alone. Furthermore, Sema3A inhibited EC migration in a Boyden chamber assay in an NRP-dependent manner and also inhibited capillary tube outgrowth in an aortic ring *in vitro* angiogenesis assay. Sema3A induced the rapid retraction of EC lamellipodia, and depolymerized F-actin, thereby collapsing the EC cytoskeleton. These effects of Sema 3A on EC appear to mimic changes that occur in cytoskeleton when Sema3A acts to collapse DRG growth cones.

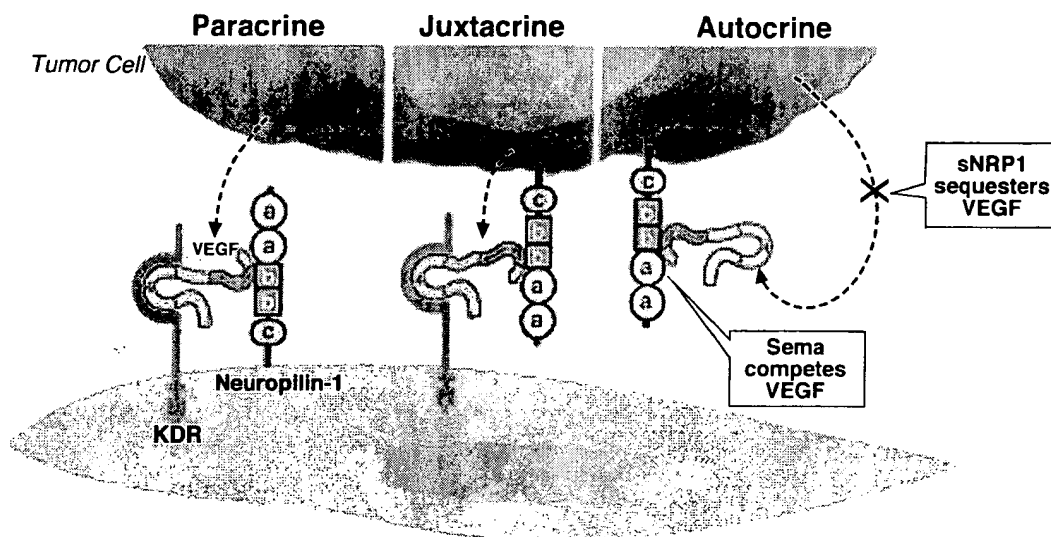


Figure 2. NRP mediates tumor angiogenesis. (Left) Paracrine interactions: tumor cells release VEGF₁₆₅, which binds to EC VEGFR-2 (KDR) via the exon 4 domain and to EC NRP via the exon 7 domain. Thus VEGF₁₆₅ bridges the two receptors on the same cell. This may be a mechanism by which NRP1 acts as a co-receptor to enhance VEGFR-2-mediated VEGF₁₆₅ activity. (Center) Juxtacrine interactions: tumor cells release VEGF₁₆₅ which binds to VEGFR-2 on EC and to NRP1 on tumor cells. As a consequence, VEGF₁₆₅ forms a bridge between the two cell types. This may be a mechanism to enhance tumor cell/EC interactions. (Right) Autocrine interactions: tumor cells release VEGF₁₆₅ which binds to tumor cell NRP1. This may be a mechanism to concentrate VEGF₁₆₅ in the vicinity of tumor cells or for VEGF₁₆₅ to signal tumor cells directly. Also depicted are two inhibitors of VEGF function. (i) sNRP1 binds to VEGF₁₆₅, inhibits its binding to EC and tumor cells, and inhibits VEGF₁₆₅ biological activity. (ii) Sema3A competes with VEGF₁₆₅ binding to NRP1, probably due to overlapping binding sites in the b domain.

VEGF₁₆₅ and Sema3A are competitive inhibitors of each other in binding, EC motility, and DRG neuron collapse assays [68], suggesting that the two ligands have overlapping NRP1-binding sites, probably the b domain to which they both bind. Thus semaphorins may have the potential of being angiogenesis inhibitors acting by dual mechanisms, one being direct inhibition of EC migration, and the other being a competitive inhibitor of VEGF₁₆₅.

VI. NRP1 and sNRP1 regulate tumor angiogenesis

An unanticipated result was that tumor cells express NRP1 and bind VEGF₁₆₅ via that receptor [4]. Some tumor cell lines express abundant NRP1, for example, prostate and breast carcinoma cell lines possess $1-2 \times 10^5$ NRP1 receptors per cell. The K_d of VEGF₁₆₅ binding to NRP1 in tumor cells is 2.8×10^{-10} M [4], approximately the same as for Sema3A binding to DRG neuron NRP1 [5]. Unlike EC, the tumor cell lines that we have examined do not express VEGFR-1

or VEGFR-2, and VEGF₁₆₅ binding to these cells is mediated solely by NRPs. NRP1 is also expressed by many tumors *in vivo*, including prostate [69], pituitary [70], squamous cell carcinoma and others (Bielenberg and Klagsbrun, unpublished). Tumor cells also express semaphorins. For example, Sema3A is expressed in various tumor cell lines (Choi, Miao, and Klagsbrun, unpublished). Sema3C and Sema3F are expressed in lung tumors [71,72], and Sema3E is expressed in mammary adenocarcinoma cells [73].

Since the function of tumor-derived NRP1 is not known, we overexpressed NRP1 in Dunning rat prostate carcinoma AT2.1 cells using a tetracycline-inducible promoter [74]. Basal cell motility and VEGF₁₆₅ binding were increased *in vitro* concomitant with induced NRP1 expression. However, induction of NRP1 did not affect tumor cell proliferation. Induction of NRP1 in AT2.1 tumor cells *in vivo* resulted in markedly increased AT2.1 cell tumor size. The tumors with induced NRP1 expression were characterized by increased microvessel density, increased EC proliferation, dilated blood vessels, and notably less tumor

cell apoptosis. Immunohistochemical analysis showed that the tumors expressing NRP1 had higher levels of VEGF protein than the control tumors, suggesting that NRP1 might retain tumor cell VEGF and prevent its diffusion out of the tumor. It was concluded that NRP1 gene expression resulted in enlarged tumors associated with substantially enhanced tumor angiogenesis [74].

On the other hand, sNRP1 appears to be anti-angiogenic. sNRP1 is secreted by cells as a 90 kDa protein corresponding to the NRP1 a and b domains [34]. It inhibits VEGF₁₆₅ binding to EC and tumor cells and inhibits VEGF₁₆₅-induced tyrosine phosphorylation of KDR in EC. Tumors of rat prostate carcinoma cells expressing recombinant sNRP1 were characterized by extensive hemorrhage, damaged vessels and apoptotic tumor cells unlike control tumors which appeared to be relatively healthy. A possible mechanism is that sNRP1 sequesters VEGF₁₆₅, and thereby blocks VEGF₁₆₅/EC and VEGF₁₆₅/tumor interactions, resulting in tumors that are less vascularized and that contain increased apoptotic tumor cells.

A model depicting mechanisms whereby NRP mediates tumor angiogenesis is shown in Figure 2.

VII. Conclusions and future directions

A current model of VEGF-mediated VEGF tumor angiogenesis holds that tumor cells release VEGF which binds to EC via the VEGF RTKs, VEGFR-1, and VEGFR-2. As a consequence, new blood vessels develop in the tumor resulting in enhanced tumor vascularization and growth. NRP1 expression also enhances tumor angiogenesis and growth. However, NRPs differ from the VEGF RTKs in that they are expressed by both EC and tumor cells. The ability of VEGF to bind to EC but also to tumor cells suggests an expanded function for this growth factor in tumor angiogenesis and progression. There are a number of challenges for the future in understanding the mechanisms by which NRP contributes to angiogenesis: (i) The significance of VEGF₁₆₅ binding to tumor cells that do not express VEGFR-1 or VEGFR-2 needs to be determined. (ii) NRP1 does not appear to be a signaling receptor by itself and is possibly a co-receptor for a signaling receptor, perhaps plexin 1. If, and how NRP1 transmits signals to tumor and other cell types needs to be determined. (iii) While NRP1 appears to enhance angiogenesis and tumor size, its soluble extracellular domain isoform, sNRP1, appears to be an

antagonist of tumor angiogenesis and growth. NRP1 and sNRP1 are products of the same gene, and often expressed in the same tumor cell. It is not known how a balance of these two factors is regulated. (iv) Tumor cells express both VEGF and semaphorins, including Sema3A. While VEGF is an angiogenic factor, Sema3A has anti-angiogenesis properties. Since VEGF₁₆₅ and Sema3A are competitive inhibitors of each other, it would be of interest to determine whether a balance of these two factors affects, in any way, the degree of tumor angiogenesis. Other important areas to investigate include identification of the regulators of NRP gene expression, determining what genes are downstream of NRP in the angiogenesis pathway, and examining the interactions between NRP and the VEGF RTKs.

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Neuropilin-1 Is Expressed by Endothelial and Tumor Cells as an Isoform-Specific Receptor for Vascular Endothelial Growth Factor

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Summary

Vascular endothelial growth factor (VEGF), a major regulator of angiogenesis, binds to two receptor tyrosine kinases, KDR/Flk-1 and Flt-1. We now describe the purification and the expression cloning from tumor cells of a third VEGF receptor, one that binds VEGF₁₆₅ but not VEGF₁₂₁. This isoform-specific VEGF receptor (VEGF₁₆₅R) is identical to human neuropilin-1, a receptor for the collapsin/semaphorin family that mediates neuronal cell guidance. When coexpressed in cells with KDR, neuropilin-1 enhances the binding of VEGF₁₆₅ to KDR and VEGF₁₆₅-mediated chemotaxis. Conversely, inhibition of VEGF₁₆₅ binding to neuropilin-1 inhibits its binding to KDR and its mitogenic activity for endothelial cells. We propose that neuropilin-1 is a novel VEGF receptor that modulates VEGF binding to KDR and subsequent bioactivity and therefore may regulate VEGF-induced angiogenesis.

Introduction

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is a prime regulator of angiogenesis, vasculogenesis, and vascular permeability (reviewed in Klagsbrun and Soker, 1993; Dvorak et al., 1995; Ferrara and Davis-Smith, 1997). The increased expression of VEGF has been correlated with vascularization of tissues, for example, during embryogenesis (Breier et al., 1992; Millauer et al., 1993; Flamme et al., 1995) and during the female reproductive cycle (Shweiki et al., 1993). Targeted disruption of even one allele of the VEGF gene results in impaired blood vessel formation, growth retardation, and death by day 10.5 (Carmeliet et al., 1996; Ferrara et al., 1996). An important role for VEGF as a mediator of tumor angiogenesis is suggested by observations that high levels of VEGF are produced by various types of tumors (Dvorak et al., 1991; Plate et al., 1992), capillaries are clustered along VEGF-producing tumor cells (Plate et al., 1992), and tumor angiogenesis

and subsequent tumor growth are inhibited in vivo by antibodies directed against VEGF (Kim et al., 1993) by soluble VEGF receptors (Kendall and Thomas, 1993) and by expression of dominant-negative VEGF receptors (Millauer et al., 1994). These properties have made the study of VEGF function relevant and significant for the study of normal physiological angiogenesis and of angiogenesis associated with abnormal cellular growth such as occurs in cancer.

Structurally, VEGF is a 40–45 kDa homodimer with limited sequence homology to the platelet-derived growth factor (Keck et al., 1989; Tischer et al., 1989). Recently, a number of structurally homologous VEGF family members have been identified, including placenta-derived growth factor (PlGF), VEGF-B, VEGF-C, and VEGF-related protein (reviewed in Klagsbrun and D'Amore, 1996). Human VEGF monomers exist as five different isoforms of 121, 145, 165, 189, and 206 amino acids that are produced by alternative splicing from a single gene containing eight exons (Tischer et al., 1991; Poltorak et al., 1997). VEGF₁₂₁ and VEGF₁₆₅ appear to be the most abundant of the isoforms. These two VEGF isoforms differ somewhat in their biological properties. For example, VEGF₁₆₅ but not VEGF₁₂₁ binds to cell surface heparan sulfate proteoglycan (HSPG), with the result that VEGF₁₆₅ is partially retained by cells while VEGF₁₂₁ is totally released (Houck et al., 1992). In addition, VEGF₁₆₅ is a more potent endothelial cell mitogen than VEGF₁₂₁ (Keyt et al., 1996b; Soker et al., 1997).

VEGF activities are mediated by high affinity tyrosine kinase receptors associated with endothelial cells (EC). Two such VEGF receptors have been identified: the 180 kDa fms-like tyrosine kinase (Flt-1) (Shibuya et al., 1990; de Vries et al., 1992) and the 200 kDa kinase insert domain-containing receptor (KDR) (Terman et al., 1992) and its murine homolog, Flk-1 (Millauer et al., 1993). KDR binds VEGF and VEGF-C, whereas Flt-1 binds VEGF and PlGF. A third structurally related tyrosine kinase receptor, 180 kDa Flt-4, binds VEGF-C but not VEGF (Joukov et al., 1996). KDR appears to be the major transducer of VEGF signals in EC that result in chemotaxis, mitogenicity, actin reorganization, and gross morphological changes in target cells (Waltenberger et al., 1994; Yoshida et al., 1996; Ferrara and Davis-Smith, 1997). Targeted disruption of the *KDR/Flk-1* gene in mice results in a defect in the development of EC and embryonic death by day 9.5 (Shalaby et al., 1995). Although Flt-1 has a higher affinity for VEGF than KDR and is phosphorylated in response to VEGF, no mitogenic response is generated (Waltenberger et al., 1994). Nevertheless, Flt-1 appears to be an important mediator of angiogenesis. It is localized to the endothelium in adult tissue (Peters et al., 1993), and targeted disruption of this receptor gene, while showing normal EC formation, results in impaired EC assembly into blood vessels and embryonic lethality (Fong et al., 1995).

VEGF has been typically considered to be an EC-specific growth factor (Keck et al., 1989; Leung et al., 1989; Plouet et al., 1989). However, there have been several reports showing VEGF interactions with non-EC.

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For example, VEGF binds to Balb/C 3T3, HeLa, and melanoma cells (Gitay-Goren et al., 1992; Enomoto et al., 1994; Soker et al., 1996). It also stimulates the migration of monocytes and osteoblasts (Midy and Plouet, 1994; Barleon et al., 1996). Cell surface receptor cross-linking experiments with ^{125}I -VEGF have suggested that additional VEGF receptors might exist that are neither KDR nor Flt-1 (Gitay-Goren et al., 1996; Soker et al., 1996; Omura et al., 1997). Recently, we identified and characterized a 130–135 kDa VEGF receptor that is different from KDR or Flt-1 (Soker et al., 1996). Interestingly, this VEGF receptor is not only associated with EC but with the cell surface of tumor cells as well, e.g., breast carcinoma, prostate carcinoma, and melanoma. It has a K_d of about $2\text{--}3 \times 10^{-10}\text{ M}$ and is expressed in relatively high copy number, 2.5×10^4 receptors/cell for EC and $1\text{--}2 \times 10^5$ receptors/cell for MDA-MB-231 (231) breast carcinoma cells (Soker et al., 1996). A striking feature of this receptor is that it binds VEGF₁₆₅ but not VEGF₁₂₁, making it isoform-specific, and we thus named it VEGF₁₆₅ receptor (VEGF₁₆₅R). The structural difference between VEGF₁₆₅ and VEGF₁₂₁ is the 44 amino acids encoded by VEGF exon 7. Recently, we demonstrated that a fusion protein containing the exon 7-encoded domain of VEGF₁₆₅ bound VEGF₁₆₅R directly and, furthermore, that it inhibited ^{125}I -VEGF₁₆₅ binding to VEGF₁₆₅R on EC and 231 cells (Soker et al., 1996, 1997). Thus, the binding of VEGF₁₆₅ to VEGF₁₆₅R occurs via VEGF exon 7 in contrast to KDR and Flt-1, which bind VEGF₁₆₅ via VEGF exons 4 and 3, respectively (Key et al., 1996a). The fusion protein also inhibited VEGF₁₆₅-mediated EC proliferation (Soker et al., 1997).

Given these results, we set out to purify and clone VEGF₁₆₅R. In this report, we demonstrate by protein purification and concomitant expression cloning that 231 cell-derived VEGF₁₆₅R is identical to human neuropilin-1, a cell surface glycoprotein that has been previously associated with axonal guidance in the developing nervous system (Kawakami et al., 1995; Takagi et al., 1995) and that recently has been demonstrated to be the receptor for the collapsin/semaphorin family of proteins (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). A second cDNA with 47% homology to *neuropilin-1* (*neuropilin-2*) was also discovered by expression cloning. Transfection of *neuropilin-1* cDNA into cells lacking endogenous VEGF receptors resulted in expression of a 130–140 kDa receptor that binds VEGF₁₆₅ but not VEGF₁₂₁. Furthermore, neuropilin-1 appears to act as a coreceptor that enhances VEGF₁₆₅ binding to KDR and VEGF₁₆₅ chemotactic and mitogenic activity. We conclude that VEGF₁₆₅R is neuropilin-1 and that this receptor may have a novel role in VEGF-associated EC and tumor biology.

Results

Purification of VEGF₁₆₅R

Cross-linking of ^{125}I -VEGF₁₆₅ to cell surface receptors of 231 breast carcinoma cells results in formation of a single 165–175 kDa labeled complex with VEGF₁₆₅R (Figure 1A, lane 1) (Soker et al., 1996). These cells have about $1\text{--}2 \times 10^5$ VEGF₁₆₅-binding sites/cell. In contrast to VEGF₁₆₅, VEGF₁₂₁ does not bind to the 231 cells and

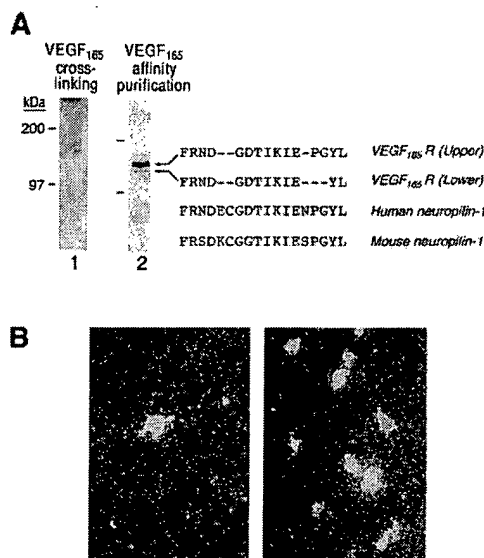


Figure 1. Purification and Expression Cloning of VEGF₁₆₅R from 231 Cells

(A) Purification. ^{125}I -VEGF₁₆₅ (5 ng/ml) was bound and cross-linked to receptors on 231 cells and analyzed by SDS-PAGE and autoradiography (lane 1). VEGF₁₆₅R was purified by Con A and VEGF₁₆₅ affinity chromatography and analyzed by SDS-PAGE and silver stain (lane 2). Two prominent bands were detected (arrows) and N-terminally sequenced separately. Their N-terminal 18-amino acid sequences are shown to the right of the arrows. The published N-terminal sequences of human and mouse neuropilin (Kawakami et al., 1995; He and Tessier-Lavigne, 1997) are shown below the VEGF₁₆₅R sequences.

(B) Expression Cloning. Photomicrographs (dark-field illumination) of COS 7 cells binding ^{125}I -VEGF₁₆₅. ^{125}I -VEGF₁₆₅ was bound to transfected COS 7 cells, which were then washed, fixed, and overlaid with photographic emulsion that was developed as described in Experimental Procedures. Left, COS 7 cells were transfected with a primary plasmid pool (number 55 of the 231 cell library) representing approximately 3×10^3 clones, and one COS 7 cell binding ^{125}I -VEGF₁₆₅ in the first round of screening is shown. Right, several COS 7 cells transfected with a single-positive cDNA clone (A2) binding ^{125}I -VEGF₁₆₅ after the third round of screening.

does not form a ligand-receptor complex (Soker et al., 1996). The relatively high VEGF₁₆₅R number and the lack of any detectable KDR or Flt-1 mRNA in 231 cells (data not shown) suggested that these cells would be useful for VEGF₁₆₅R purification. Preliminary characterization indicated that VEGF₁₆₅R was a glycoprotein. A combination of Con A Sepharose and VEGF₁₆₅-Sepharose affinity chromatography was used for the purification of VEGF₁₆₅R as described in Experimental Procedures. A prominent doublet with a molecular mass of about 130–135 kDa was detected by SDS-PAGE and silver stain (Figure 1A, lane 2). This size is consistent with the formation of a 165–175 kDa complex of 40–45 kDa VEGF₁₆₅ bound to receptors approximately 130–135 kDa in size (Figure 1A, lane 1). The two bands were excised separately and N-terminal amino acid sequencing was carried out (Figure 1A, right). Both the upper and lower bands had similar N-terminal amino acid sequences and showed high degrees of sequence homology to the predicted amino acid sequences in the N-terminal regions of

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1  MERGLPFLCAVLALVLAFAAERNDKCGDTIKIESPGYLTSPGYPHSYHPSEKCEWLIQAPDPYQIRIMIN 70
71  FNPBFDLEDRCRKYDYVEVFDGENENGHFRGKFCGKIAPPPVSSGPFLLFKIPVSDYETHGAGFSIRYEI 140
141  PKRGPECSQNYTTPSGVKSPPGFPEXYPNSLECTYIVFAPKMSBILILEPESFDLEPDSNPPGGHPCRYDR 210
211  LEIWDFPDVGPVHIGRYCGQKTPGIRSSSSGILSMVFTDSIAKEGFSANYSVLQSSVSEDFRCMEALG 280
281  MESGEINSDQITASSQYSTNWSAERSRLNYPENGWTPGEDSYREWIQVDLGLRFTAVGTQGAISKETK 350
351  KKYVYKTYKIDVSSNGEDWITIREGNKPVLFQGNNTPTOVVVAVFPKPLITRFVRIKPATWETGISMRF 420
421  VYGGKITDYPSCGMLGMSGLISDSQITSSNQGRNWNPNIRLVTSSRGWALPAPHSYINELWQIDLG 490
491  EEKIVRGIIQGGKHRENKVFMRKPKIGYSNNGSDWKMIMDDSKRKAKEPFGNNNYDTPELRTFFALSTR 560
561  FIRIYPERATHGGLGLRMELLGCEVEAPTAGPTTPNGNLVDZCDDQANCSHSGTGDDFQLTGGTTVLATE 630
631  KPTVIDSTIQSEFPPTYGFNCEFGWGSNKTFCHEWEDNHVQLKNSVLTSTKTCFIQDHTGDNFIYSQADEN 700
701  QKGKVARLVSPVVYSQNSAHCMTFWYHMSGSHVGTLRVKLRYPKEEYDQVWMAIGHQGDHWEKGRVLL 770
771  HSKLKYQVIFEGEIGKGNLGGIAVDDISINNHISQEDCAKPAOLDKKNPEIKIDETGCTPGYEGEGEGD 840
841  KNISRKPGNVLTLDPLITIANSSALGVLLGAVCGVVLVYCAQHNGMSERNLSALENYNFELVDGVKLG 910
911  KDKLNTQSTYSEA 923

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Figure 2. The Deduced Amino Acid Sequence of Human *neuropilin-1*

The deduced 923-amino acid sequence of the ORF of *neuropilin-1* clone A2 (full insert size of 6.5 kb) is shown. The putative signal peptide sequence (amino acids 1–21) and the putative transmembrane region (amino acids 860–883) are in boxes. The amino acid sequence obtained by N-terminal amino acid sequencing (Figure 1A, amino acids 22–39) is underlined. The arrow indicates where the signal peptide has been cleaved and removed based on comparison of the N-terminal sequence of purified *neuropilin-1* and the cDNA sequence. The sequence of human *neuropilin-1* reported here differs from that reported by He and Tessier-Lavigne (1997) in that we find Lys26 rather than Glu26 and Asp855 rather than Glu855. Lys26 and Asp855 are found, however, in mouse and rat *neuropilin-1* (Kawakami et al., 1995; He and Tessier-Lavigne, 1997).

mouse (Kawakami et al., 1995) and human (He and Tessier-Lavigne, 1997) *neuropilin-1*.

Expression Cloning of VEGF₁₆₅R from 231 Cell-Derived mRNA

Concomitant with the purification, expression cloning was used to clone VEGF₁₆₅R. The details of these experiments are described in Experimental Procedures. For expression cloning, 231 cell mRNA was used to prepare a cDNA library of approximately 10⁷ clones in a eukaryotic expression plasmid. Pools of this library were transfected into COS-7 cells in separate wells, and individual cells were screened for the ability to bind [¹²⁵I]-VEGF₁₆₅ as detected by autoradiography of monolayers overlaid with photographic emulsion (Figure 1B, left). After three rounds of subpooling and screening, seven single-positive cDNA clones were obtained. Figure 1B (right) shows binding of [¹²⁵I]-VEGF₁₆₅ to COS-7 cells transfected with one of these single-positive clones (clone A2).

Restriction enzyme analysis revealed that six of the seven single-positive clones had identical restriction digestion patterns, but one clone had a pattern that was different (data not shown). Sequencing of one of the identical cDNA clones, clone A2 (Figure 2), showed it to be identical to a sequence derived from a human-expressed sequence tag data bank (dbEST). This sequence also showed a high percentage of homology to the sequence of mouse *neuropilin-1* (Kawakami et al., 1995). After we had cloned human VEGF₁₆₅R, two groups reported the cloning of rat and human receptors for semaphorin III and identified them to be *neuropilin-1* (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). The 231 cell-derived VEGF₁₆₅R cDNA sequence is virtually identical (see the Figure 2 legend for exceptions) to the human *neuropilin-1* sequence (He and Tessier-Lavigne, 1997). Significantly, the predicted amino acid sequence obtained by expression cloning (Figure 2) confirmed that VEGF₁₆₅R was *neuropilin-1* as was determined by N-terminal sequencing of purified receptor (Figure 1A). The human *neuropilin-1* cDNA sequence predicts an open reading frame (ORF) of 923 amino acids

with two hydrophobic regions representing putative signal peptide and transmembrane domains. Overall, the sequence predicts ectodomain, transmembrane, and cytoplasmic domains consistent with the structure of a cell surface receptor. The N-terminal sequence obtained via protein purification as shown in Figure 1A is downstream of a 21-amino acid putative hydrophobic signal peptide domain, thereby indicating directly where the signal peptide domain is cleaved and removed.

Sequence analysis of the one clone obtained by expression cloning that had a different restriction enzyme profile predicted an ORF of 931 amino acids with about a 47% homology to *neuropilin-1* (data not shown). This human cDNA has a 93% sequence homology with rat *neuropilin-2* and is identical to the recently cloned human *neuropilin-2* (Chen et al., 1997).

Expression of Neuropilin-1 in Adult Cell Lines and Tissues

Reports of *neuropilin-1* gene expression have been limited so far predominantly to the nervous system of the developing embryo (Takagi et al., 1987, 1995; Kawakami et al., 1995). Cell surface VEGF₁₆₅R, however, is associated with nonneuronal adult cell types such as EC and a variety of tumor-derived cells (Soker et al., 1996). Northern blot analysis was carried out to determine whether cells that bind [¹²⁵I]-VEGF₁₆₅ also synthesized *neuropilin-1* mRNA (Figure 3). *neuropilin-1* mRNA transcripts were expressed in human umbilical vein EC (HUVEC) (Figure 3A, lane 1). In tumor cell lines (Figure 3A, lanes 2–8), *neuropilin-1* mRNA levels were highest in 231 and PC3 cells and to a lesser degree in LNCaP, EP-mel, and RU-mel cells. There was little if any expression in MDA-MB-453 and WK-mel cells. The *neuropilin-1* gene expression patterns were consistent with our previous results showing that HUVEC, 231, PC3, LNCaP, EP-mel, and RU-mel cells bind [¹²⁵I]-VEGF₁₆₅ to cell surface VEGF₁₆₅R but that MDA-MB-453 and WK-mel cells do not (Soker et al., 1996).

neuropilin-1 gene expression was analyzed also by Northern blot in a variety of adult tissues in comparison to *KDR* gene expression (Figure 3B). *neuropilin-1* mRNA

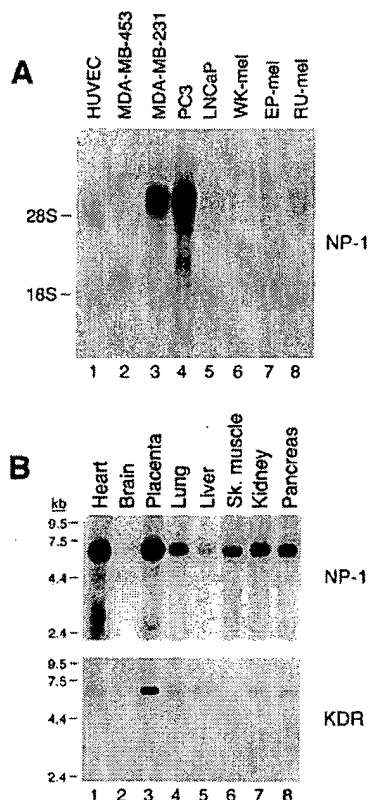


Figure 3. Northern Blot Analysis of *neuropilin-1* Expression

(A) Human EC and tumor-derived cell lines. Total RNA samples prepared from HUVEC and tumor-derived breast carcinoma, prostate carcinoma, and melanoma cell lines as indicated were resolved on a 1% agarose gel and blotted onto a GeneScreen nylon membrane. The membrane was probed with ^{32}P -labeled *neuropilin-1* cDNA and exposed to X-ray film. Equal RNA loading was demonstrated by ethidium bromide staining of the gel prior to blotting. A major species of *neuropilin-1* mRNA of approximately 7 kb was detected in several cell lines.

(B) Adult human tissues. A premade Northern blot membrane containing multiple samples of human mRNA (Clontech) was probed with ^{32}P -labeled *neuropilin-1* cDNA (top) and then stripped and re-probed with ^{32}P -labeled *KDR* cDNA (bottom).

levels were relatively high in heart and placenta; more moderate in lung, liver, skeletal muscle, kidney, and pancreas; and relatively low in adult brain. Interestingly, previous analysis of *neuropilin-1* gene expression in mouse and chicken brain suggested that this gene was expressed primarily during embryonic development and was greatly diminished after birth (Takagi et al., 1995; Kawakami et al., 1995). The tissue distribution of *KDR* mRNA was similar to that of *neuropilin-1*, with the exception that it was not expressed as highly in the heart. These results indicate that *neuropilin-1* is expressed widely in adult nonneuronal tissue, including tissues in which angiogenesis occurs such as heart and placenta.

Characterization of VEGF₁₆₅ Binding to Neuropilin-1

In order to characterize the binding properties of neuropilin-1, porcine aortic endothelial (PAE) cells were transfected with *neuropilin-1* cDNA. The PAE cells were

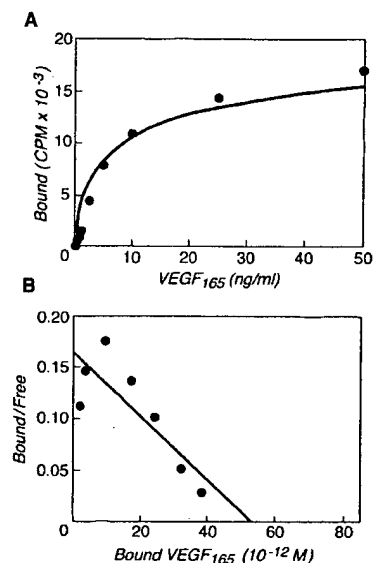


Figure 4. Scatchard Analysis of VEGF₁₆₅ Binding to Neuropilin-1

(A) Increasing amounts of ^{125}I -VEGF₁₆₅ (0.1–50 ng/ml) were added to subconfluent cultures of PAE cells transfected with human *neuropilin-1* cDNA (PAE/NP-1 cells) in 48-well dishes. No exogenous heparin was added. After binding the cells were washed, lysed, and the cell-associated radioactivity was determined using a γ counter. Nonspecific binding was determined by competition with a 200-fold excess of unlabeled VEGF₁₆₅.

(B) The binding data shown in (A) were analyzed by the method of Scatchard, and a best fit plot was obtained with the Ligand program (Munson and Rodbard, 1980). PAE/NP-1 cells express approximately 4.5×10^4 VEGF₁₆₅-binding sites per cell and bind ^{125}I -VEGF₁₆₅ with a K_d of 3×10^{-10} M.

chosen for these expression studies because they express neither KDR, Flt-1, (Waltenberger et al., 1994), nor VEGF₁₆₅R. Stable cell lines synthesizing neuropilin-1 (PAE/NP-1) were established, and ^{125}I -VEGF₁₆₅ binding experiments were carried out (Figure 4). ^{125}I -VEGF₁₆₅ binding to PAE/NP-1 cells increased in a dose-dependent manner and reached saturation at approximately 30 ng/ml (Figure 4A). Scatchard analysis of VEGF₁₆₅ binding revealed a single class of VEGF₁₆₅-binding sites with a K_d of approximately 3.2×10^{-10} M and approximately 4.5×10^4 binding sites per cell (Figure 4B). Similar K_d values were obtained for several PAE/NP-1 clones generated independently, although the receptor number varied from clone to clone (data not shown). The K_d of 3.2×10^{-10} M for the PAE/NP-1 cell lines is consistent with the $2\text{--}2.8 \times 10^{-10}$ M K_d values obtained for VEGF₁₆₅R expressed naturally by HUVEC and 231 cells (Soker et al., 1996). The binding of ^{125}I -VEGF₁₆₅ to PAE/NP-1 cells was enhanced by 1 $\mu\text{g/ml}$ heparin (data not shown), consistent with previous studies showing that heparin enhances ^{125}I -VEGF₁₆₅ binding to VEGF₁₆₅R on HUVEC and 231 cells (Gitay-Goren et al., 1992; Soker et al., 1996).

Isoform-Specific Binding of VEGF to Cells Expressing Neuropilin-1

VEGF₁₆₅ but not VEGF₁₂₁, binds to VEGF₁₆₅R on HUVEC and 231 cells (Gitay-Goren et al., 1996; Soker et al., 1996).

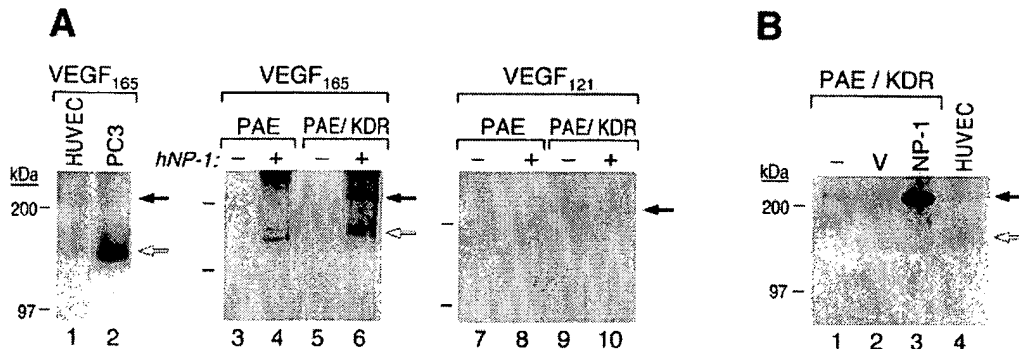


Figure 5. Cross-linking of VEGF Isoforms to PAE Cells Expressing Neuropilin-1, KDR, or Both Receptors

Stable transfections. ^{125}I -VEGF₁₆₅ (5 ng/ml) (lanes 1–6) or ^{125}I -VEGF₁₂₁ (10 ng/ml) (lanes 7–10) were bound to subconfluent cultures of HUVEC (lane 1), PC3 cells (lane 2), PAE cells (lanes 3 and 7), PAE cells transfected with human *neuropilin-1* cDNA (PAE/NP-1) (lanes 4 and 8), PAE cells transfected with *KDR* cDNA (PAE/KDR) (lanes 5 and 9), and PAE/KDR cells transfected with human *neuropilin-1* cDNA (PAE/KDR/NP-1) (lanes 6 and 10). At the end of a 2 hr incubation, each ^{125}I -VEGF isoform was chemically cross-linked to the cell surface. The cells were lysed and proteins were resolved by 6% SDS-PAGE. The polyacrylamide gel was dried and exposed to X-ray film. Solid arrows denote radiolabeled complexes containing ^{125}I -VEGF and KDR; open arrows denote radiolabeled complexes containing ^{125}I -VEGF₁₆₅ and neuropilin-1. (B) Transient transfections. PAE/KDR cells were transfected with pCPhyg or pCPhyg-NP-1 plasmids as described in Experimental Procedures and grown for 3 days in 6 cm dishes. ^{125}I -VEGF₁₆₅ (5 ng/ml) was bound and cross-linked to parental PAE/KDR cells (lane 1), PAE/KDR cells transfected with vector control (V) (lane 2), PAE/KDR cells transfected with pCPhyg-NP-1 plasmids (NP-1) (lane 3), and HUVEC (lane 4). The cells were lysed and proteins were resolved by 6% SDS-PAGE as (A). Solid and open arrows denote labeled complexes as in (A).

To ascertain whether cells transfected with *neuropilin-1* had the same isoform binding specificity, PAE/NP-1 cells were incubated with ^{125}I -VEGF₁₆₅ or ^{125}I -VEGF₁₂₁ followed by cross-linking (Figure 5A). ^{125}I -VEGF₁₆₅ did not bind to parental PAE cells (Figure 5A, lane 3) but did bind to PAE/NP-1 cells via neuropilin-1 (Figure 5A, lane 4). The radiolabeled complexes formed with neuropilin-1 were similar in size to those formed in PC3 cells (Figure 5A, lane 2). On the other hand, ^{125}I -VEGF₁₂₁ did not bind to parental PAE (Figure 5A, lane 7) or to PAE/NP-1 cells (Figure 5A, lane 8). These results demonstrate that the VEGF isoform-specific binding that occurs with cells expressing endogenous neuropilin-1 (VEGF₁₆₅R) such as HUVEC, 231, and PC3 cells can be replicated in cells transfected with *neuropilin-1* cDNA and support the finding that VEGF₁₆₅R and neuropilin-1 are identical.

Coexpression of Neuropilin-1 and KDR Modulates VEGF₁₆₅ Binding to KDR

To determine whether expression of neuropilin-1 had any effect on VEGF₁₆₅ interactions with KDR, PAE cells that were previously transfected with *KDR* cDNA to produce stable clones of PAE/KDR cells (Waltenberger et al., 1994) were transfected with *neuropilin-1* cDNA, and stable clones expressing both receptors (PAE/KDR/NP-1) were obtained. These cells bound ^{125}I -VEGF₁₆₅ to KDR (Figure 5A, lane 6, upper complex) and to neuropilin-1 (Figure 5A, lane 6, lower complex) to yield a cross-linking profile similar to HUVEC (Figure 5A, lane 1). On the other hand, PAE/KDR/NP-1 cells bound ^{125}I -VEGF₁₂₁ to form a complex only with KDR (Figure 5A, lane 10), consistent with the inability of VEGF₁₂₁ to bind to neuropilin-1.

It appeared that in cells coexpressing KDR and neuropilin-1 (Figure 5A, lane 6) the degree of ^{125}I -VEGF₁₆₅-KDR 240 kDa complex formation was enhanced about 4-fold compared to the parental PAE/KDR cells (Figure 5A, lane 5). These results were reproducible, and the degree of 240 kDa complex formation in different PAE/

KDR/NP-1 clones correlated positively with the levels of neuropilin-1 expressed. Binding experiments were carried out to quantitate these results (data not shown). Increasing concentrations of ^{125}I -VEGF₁₆₅ were added to PAE/KDR and PAE/KDR/NP-1 cells. At 30 ng/ml, the levels of ^{125}I -VEGF₁₆₅ bound to PAE/KDR/NP-1 cells were about 6-fold higher than to PAE/KDR cells, consistent with the cross-linking data shown in Figure 5. However, accurate measurement of the individual affinities (K_d s) of VEGF₁₆₅ binding to KDR and neuropilin-1 when they were coexpressed in PAE cells was difficult because the K_d s of KDR (Waltenberger et al., 1994; our results) and neuropilin-1 (Figure 4), when expressed independently in PAE cells, are fairly similar.

It could not be ruled out definitively that the enhanced binding to KDR was possibly due to clonal selection posttransfection. Therefore, parental PAE/KDR cells were transfected with *neuropilin-1* cDNA and ^{125}I -VEGF₁₆₅ was bound and cross-linked to the cells 3 days later in order to avoid selecting clones that might have shown diversity in KDR expression (Figure 5B). A labeled 240 kDa complex containing KDR was formed in parental PAE/KDR cells (Figure 5B, lane 1) and in PAE/KDR cells transfected with the expression vector alone (Figure 5B, lane 2). However, when ^{125}I -VEGF₁₆₅ was cross-linked to PAE/KDR cells expressing neuropilin-1 transiently, a more intensely labeled 240 kDa complex, about four times greater, was observed (Figure 5B, lane 3), compared to parental PAE/KDR cells (Figure 5B, lane 1) and PAE/KDR cells transfected with expression vector (Figure 5B, lane 2). Taken together, the results shown in Figures 5A and 5B suggest that coexpression of *KDR* and *neuropilin-1* genes in the same cell enhances the ability of VEGF₁₆₅ to bind to KDR.

Coexpression of Neuropilin-1 and KDR Enhances VEGF₁₆₅ Chemotactic Activity

To determine whether neuropilin-1 expression modulated VEGF₁₆₅ bioactivity, PAE, PAE/NP-1, PAE/KDR,

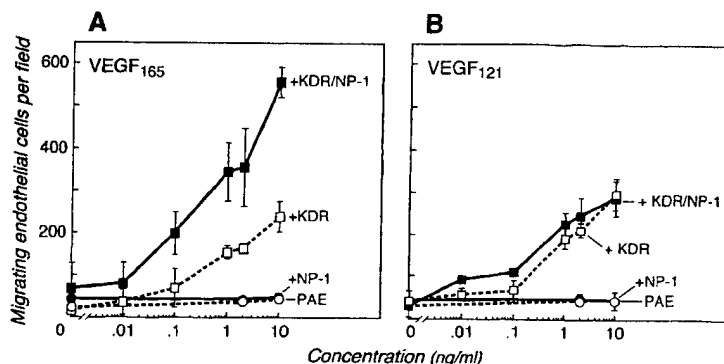


Figure 6. VEGF-Induced Chemotaxis in PAE Cells Expressing Neuropilin-1, KDR, or Both Receptors

Chemotaxis of PAE cells (open circles), PAE cells expressing neuropilin-1 (closed circles), PAE cells expressing KDR (open boxes), and PAE cells expressing both neuropilin-1 and KDR (closed boxes) toward increasing concentrations of VEGF₁₆₅ (A) or VEGF₁₂₁ (B) was measured in a Boyden chamber as described in Experimental Procedures. Each point represents the mean cell number and standard deviations of four independent wells.

and PAE/KDR/Neuropilin-1 cells were analyzed for their chemotaxis toward gradients of VEGF₁₆₅ or VEGF₁₂₁ in a Boyden chamber assay (Figure 6). There was no chemotaxis of parental PAE or PAE-NP-1 toward either VEGF isoform. Both these cell types were viable, however, and migrated well toward 10% serum (data not shown). Both VEGF₁₆₅ (Figure 6A) and VEGF₁₂₁ (Figure 6B) stimulated PAE/KDR cell migration in a dose-response manner and, to a similar extent, about 8-fold above control at 10 ng/ml VEGF. However, when PAE/KDR/Neuropilin-1 cells were examined, a notable difference in chemotaxis was observed. VEGF₁₆₅ at 10 ng/ml stimulated a 2.5-fold greater migration of PAE/KDR/Neuropilin-1 cells compared to PAE/KDR cells, while VEGF₁₂₁ did not induce any enhanced migration. Checkerboard analysis (Yoshida et al., 1996; Elenius et al., 1997) confirmed that the motility of the cell types was due to chemotaxis (data not shown). It was concluded that coexpression of neuropilin-1 with KDR not only results in increased VEGF₁₆₅ binding to KDR but in increased chemotaxis as well.

A GST-VEGF Exon 7+8 Fusion Protein Inhibits VEGF₁₆₅ Binding to VEGF Receptors and Mitogenicity for HUVEC

We have previously shown that ¹²⁵I-VEGF₁₆₅ binds to VEGF₁₆₅R through its exon 7-encoded domain and that a GST fusion protein containing the peptide encoded by VEGF exon 7+8 (GST-Ex 7+8) inhibits completely the binding of ¹²⁵I-VEGF₁₆₅ to VEGF₁₆₅R associated with 231 cells and HUVEC (Soker et al., 1996, 1997). When added to PAE/Neuropilin-1 cells, the fusion protein completely inhibited binding to neuropilin-1 (Figure 7A, lane 2 compared to lane 1). On the other hand, it did not inhibit ¹²⁵I-VEGF₁₆₅ binding to KDR (Figure 7A, lane 4 compared to lane 3). Thus, these results demonstrate that GST-Ex 7+8 binds to neuropilin-1 but not to KDR. The effects of GST-Ex 7+8 were different, however, in cells coexpressing both neuropilin-1 and KDR. The degree of ¹²⁵I-VEGF₁₆₅ binding to KDR in PAE/KDR/Neuropilin-1 cells (Figure 7A, lane 5) was greater than to the parental PAE/KDR cells (Figure 7A, lane 3), consistent with the results in Figure 5. In PAE/KDR/Neuropilin-1 cells, GST-Ex 7+8 inhibited not only ¹²⁵I-VEGF₁₆₅ binding to neuropilin-1 completely, but, unexpectedly, it also inhibited binding to KDR substantially (Figure 7A, lane 6 compared to lane 5). In fact, in the presence of GST-Ex 7+8, binding of ¹²⁵I-VEGF₁₆₅ to KDR in PAE/KDR/Neuropilin-1 cells was reduced

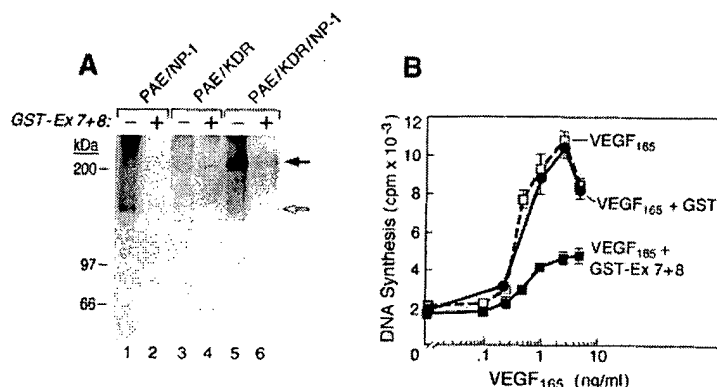
to the levels seen in parental PAE/KDR cells not expressing neuropilin-1 (Figure 7A, lane 6 compared to lane 4). Similar inhibition of ¹²⁵I-VEGF₁₆₅ binding and cross-linking to KDR by GST-Ex 7+8 was observed in HUVEC, which express both KDR and neuropilin-1 (Soker et al., 1997). Since the fusion protein does not bind to KDR, these results suggest that inhibiting the binding of ¹²⁵I-VEGF₁₆₅ to neuropilin-1 directly inhibits its binding to KDR indirectly.

To determine whether the inhibitory effects of GST-Ex 7+8 on VEGF₁₆₅ binding also affected VEGF-induced mitogenicity, HUVEC were incubated with VEGF₁₆₅ in the presence or absence of GST-Ex 7+8 (Figure 7B). GST-Ex 7+8 inhibited VEGF₁₆₅-induced DNA synthesis by about 2- to 3-fold, confirming previous results (Soker et al., 1997). GST alone did not affect HUVEC proliferation. These results suggest that blocking VEGF₁₆₅ access to neuropilin-1 inhibits VEGF₁₆₅ mitogenic activity.

Discussion

Neuropilin-1, a Mediator of Neuronal Guidance, Is an Isoform-Specific VEGF₁₆₅ Receptor

Recently, we described a novel 130–135 kDa VEGF cell surface receptor that binds VEGF₁₆₅ but not VEGF₁₂₁ and that we named, accordingly, VEGF₁₆₅R (Soker et al., 1996). We have now purified VEGF₁₆₅R, expression cloned its cDNA, and shown it to be identical to human neuropilin-1 (He and Tessier-Lavigne, 1997), a 130–140 kDa cell surface glycoprotein first identified in *Xenopus* tadpole nervous tissue (Takagi et al., 1987). The evidence that VEGF₁₆₅R is identical to neuropilin-1 and that neuropilin-1 serves as a receptor for VEGF₁₆₅ is as follows: (i) Purification of VEGF₁₆₅R protein from human MDA-MB-231 (231) cells using VEGF affinity yielded a 130–140 kDa doublet upon SDS-PAGE and silver stain. N-terminal sequencing of both proteins yielded the same N-terminal sequence of 18 amino acids that demonstrated a high degree of homology to mouse and human neuropilin-1 (Kawakami et al., 1995; He and Tessier-Lavigne, 1997). The molecular mass of VEGF₁₆₅R is about 130–140 kDa, consistent with the size of neuropilin-1. (ii) Expression cloning using a 231 cell cDNA library resulted in isolation of several cDNA clones whose sequences were identical to that of human neuropilin-1 cDNA (He and Tessier-Lavigne, 1997). The combination of purification and expression cloning has the advantage



(A) Inhibition of binding. 125 I-VEGF₁₆₅ (5 ng/ml) was bound to subconfluent cultures of PAE transfected with *neuropilin-1* cDNA (PAE/NP-1) (lanes 1 and 2), PAE cells transfected with KDR (PAE/KDR) (lanes 3 and 4), and PAE/KDR cells transfected with *neuropilin-1* cDNA (PAE/KDR/NP-1) (lanes 5 and 6) in 35 mm dishes. The binding was carried out in the presence (lanes 2, 4, and 6) or absence (lanes 1, 3, and 5) of 25 μ g/ml GST-Ex 7+8. 125 I-VEGF₁₆₅ was chemically cross-linked to the cell surface. Cells were lysed and labeled proteins were resolved by SDS-PAGE as in Figure 5. Solid and open arrows denote radiolabeled complexes as in Figure 5.

(B) Inhibition of proliferation. HUVEC were cultured in 96-well dishes (5000 cells/well) with increasing concentrations of VEGF₁₆₅ (open squares), VEGF₁₆₅ + 25 μ g/ml of GST-Ex 7+8 (closed squares), or VEGF₁₆₅ + 30 μ g/ml GST alone (closed circles). After a 4 day incubation, incorporation of 3 H-thymidine into HUVEC was measured. The results represent the average count of three wells, and the standard deviations were determined.

of allowing unambiguous identification of the neuropilin-1 protein N terminus. (iii) Northern blot analysis of *neuropilin-1* gene expression was consistent with previous 125 I-VEGF₁₆₅ cross-linking experiments (Soker et al., 1996). (iv) When neuropilin-1 was expressed in PAE cells, the transfected cells were able to bind VEGF₁₆₅ but not VEGF₁₂₁, consistent with the isoform specificity of binding previously shown for HUVEC and 231 cells (Soker et al., 1996). Furthermore, the K_d of 125 I-VEGF₁₆₅ binding to PAE expressing neuropilin-1 was about 3×10^{-10} M, consistent with previous VEGF₁₆₅R K_d binding values of 2 and 2.8×10^{-10} M for HUVEC and 231 cells, respectively (Soker et al., 1996). Taken together, these results show that VEGF₁₆₅R is identical to neuropilin-1.

In addition to the expression cloning of *neuropilin-1* cDNA, another human cDNA clone was isolated whose predicted amino acid sequence was 47% homologous to that of *neuropilin-1* and over 90% homologous to rat *neuropilin-2*, which was recently cloned (Kolodkin et al., 1997). Neuropilin-2 binds members of the collapsin/semaphorin family selectively (Chen et al., 1997). Whether this type of selectivity occurs for members of the VEGF family is under investigation.

The discovery that neuropilin-1 serves as a receptor for VEGF₁₆₅, an angiogenesis factor, is surprising since, to date, neuropilin-1 has been considered to be essentially a neuronal cell surface protein that plays a role in axon growth and guidance in the developing embryo (Fujisawa et al., 1995; Kawakami et al., 1995). Our results are novel in showing that *neuropilin-1* is also expressed widely in human adult tissues. Northern blot analysis demonstrates relatively high levels of *neuropilin-1* mRNA transcripts in nonneuronal human tissues such as heart, placenta, lung, liver, skeletal muscle, kidney, and pancreas. Interestingly, there is very little relative *neuropilin-1* expression in adult brain, consistent with previous studies in the mouse nervous system showing greatly diminished *neuropilin-1* expression after birth (Kawakami et al., 1995). *neuropilin-1* is also expressed in a number of cultured nonneuronal cell lines including a variety of tumor-derived cells and EC. Expression of *neuropilin-1* by cultured EC is consistent with previous

studies demonstrating *neuropilin-1* expression in capillary EC in vivo in the embryonic mouse cardiovascular system (Kitsukawa et al., 1995).

Neuropilin-1 has been identified recently as a receptor that mediates the chemorepulsant activity of the collapsin/semaphorins (collapsin-1/Sema III/Sema D), a large family of transmembrane and secreted glycoproteins that function in repulsive growth cone and axon guidance (Kolodkin et al., 1993, 1997; He and Tessier-Lavigne, 1997). The K_d of Sema III binding to neuropilin-1, $0.15-3.25 \times 10^{-10}$ M (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997) is similar to that of VEGF₁₆₅ binding to neuropilin-1, which is about $2-3 \times 10^{-10}$ M. These results indicate that two structurally different ligands with markedly different biological activities, stimulation of angiogenesis by VEGF and chemorepulsion of neuronal cells by Sema III, bind to the same receptor and with similar affinity. An interesting question is whether the two ligands bind to the same site on neuropilin-1 or to different sites. Neuropilin-1 has five discrete domains in its ectodomain, and it has been suggested that this diversity of protein modules in neuropilin-1 is consistent with the possibility of multiple binding ligands for neuropilin-1 (Takagi et al., 1991; Feiner et al., 1997; He and Tessier-Lavigne, 1997). Preliminary analysis does not indicate any large degree of sequence homology between Sema III and VEGF exon 7, which is responsible for VEGF binding to neuropilin-1. However, there may be some three-dimensional structural similarities between the two ligands. Since both neurons and blood vessels display branching and directional migration, it would be interesting to determine whether VEGF₁₆₅ displays any neuronal guidance activity and whether Sema III has any EC chemorepulsant activity.

A Role for Neuropilin-1 in Mediating VEGF₁₆₅ Activity and Angiogenesis

An important question is whether neuropilin-1 mediates VEGF₁₆₅ activity and, by extension, angiogenesis. Several pieces of evidence presented here suggest that neuropilin-1 regulates VEGF₁₆₅/KDR interactions and VEGF₁₆₅ activity as follows: (i) Neuropilin-1 enhances the binding

of VEGF₁₆₅ to KDR. When neuropilin-1 and KDR are coexpressed in EC, cross-linking and quantitative binding studies demonstrate that the binding of ¹²⁵I-VEGF₁₆₅ to KDR in cells coexpressing neuropilin-1 is enhanced by 4- to 6-fold compared to cells expressing KDR alone. It was not possible, however, to determine accurately the effect of neuropilin-1 on KDR binding affinity due to the similarities of the individual K_ds of neuropilin-1 and KDR in these cells. Enhanced binding can be demonstrated in stable clones coexpressing neuropilin-1 and KDR and also in PAE/KDR cells transfected transiently with *neuropilin-1* cDNA where clonal selection does not take place. (ii) The chemotaxis of PAE coexpressing neuropilin-1 and KDR toward a gradient of VEGF₁₆₅ is enhanced about 2.5-fold compared to PAE expressing KDR alone. On the other hand, there is no enhancement in the chemotaxis of cells coexpressing the two receptors toward VEGF₁₂₁, consistent with the isoform-specific binding of VEGF to neuropilin-1. PAE cells expressing neuropilin-1 alone do not migrate in response to gradients of VEGF₁₆₅, suggesting that this receptor does not function by itself in mediating cell motility but acts rather as a coreceptor. (iii) Conversely, blocking VEGF₁₆₅ access to neuropilin-1 substantially inhibits VEGF₁₆₅ binding to KDR and its mitogenic activity for HUVEC. This result was achieved using a GST fusion protein containing the 44-amino acid exon 7 of VEGF, the domain responsible for VEGF₁₆₅ binding to neuropilin-1, and the 6 amino acid exon 8, which facilitates production of the fusion protein (Soker et al., 1996, 1997). GST-Ex 7+8 is not only a direct and complete inhibitor of VEGF₁₆₅ binding to neuropilin-1 in PAE/KDR/NP-1 cells, but it is also inhibits indirectly VEGF₁₆₅ binding to KDR down to the levels observed in cells expressing KDR alone. The GST-Ex 7+8 protein also inhibits VEGF₁₆₅ mitogenic activity for HUVEC by 2- to 3-fold, confirming previous studies which showed that VEGF₁₆₅-induced HUVEC proliferation was inhibited down to the level induced by VEGF₁₂₁ (Soker et al., 1997). The combination of experiments in which neuropilin-1 is either overexpressed or is inhibited suggest strongly that neuropilin-1 is functional in EC by serving as a coreceptor that enhances VEGF₁₆₅ but not VEGF₁₂₁ binding to KDR and subsequent bioactivity. The concept that dual receptors regulate growth factor binding and activity has been previously demonstrated for TGF- β , bFGF, and NGF (Yayon et al., 1991; Lopez-Casillas et al., 1993; Barbacid, 1995). Expression of betaglycan in particular has been shown to increase TGF- β binding to its signaling receptor and to enhance cell responsiveness to TGF- β (Lopez-Casillas et al., 1993). At this point, the possible effects of neuropilin-1 on mediating KDR signaling have not been determined.

Compelling evidence suggesting that neuropilin-1 is involved in mediating angiogenesis comes from previous studies in other laboratories using transgenic mice (Kitsukawa et al., 1995). In the normal mouse embryo, neuropilin-1 is expressed in EC and in mesenchymal cells surrounding the EC. However, overexpression of neuropilin-1 ectopically in transgenic mice resulted in embryonic lethality, and the mice died in utero no later than on embryonic day 17.5. In these mice, the embryos possessed excess capillaries and blood vessels that were abnormal compared to normal counterparts. Dilated blood vessels were also observed and some of

the chimeric mice showed hemorrhaging, mainly in the head and neck. These results are consistent with the possibility that ectopic overexpression of neuropilin-1 results in inappropriate VEGF₁₆₅ activity, thereby leading to aberrant angiogenesis. Furthermore, in a recent study the embryos of transgenic mice targeted for disruption of the *neuropilin-1* gene had severe abnormalities in the peripheral nervous system, but their death in utero at day 10.5–12.5 appeared to be due to anomalies in the cardiovascular system (Kitsukawa et al., 1997).

Neuropilin-1 Is Associated with Tumor-Derived Cells

The greatest degree of neuropilin-1 expression that we have detected so far occurs in tumor-derived cells such as 231 breast carcinoma cells and PC3 prostate carcinoma cells, far more than occurs in HUVEC. The tumor cells express abundant levels of *neuropilin-1* mRNA and about 200,000 VEGF₁₆₅ receptors/cell (Soker et al., 1996). On the other hand, these tumor cells do not express KDR or Flt-1, so neuropilin-1 is the only VEGF receptor associated with these cells. The role of neuropilin-1 in tumors in the absence of high affinity receptors is unknown. It may be that, besides the documented paracrine effects of tumor cell-derived VEGF on stimulating EC, VEGF₁₆₅ can affect tumor cells directly in an autocrine manner. To date, we have not been able to show VEGF₁₆₅-induced phosphorylation of neuropilin-1 in tumor-derived cells, consistent with the lack of a consensus tyrosine kinase domain. Nevertheless, many other cellular signaling mechanisms exist, and VEGF₁₆₅ might have an effect on tumor cells by inducing some as yet undetermined activity such as enhanced survival, differentiation, or motility. Another possibility is that tumor cell neuropilin-1 has a storage and sequestration function for VEGF₁₆₅. The ability of VEGF₁₆₅ but not VEGF₁₂₁ to bind to tumor cells via neuropilin-1 suggests that VEGF₁₆₅ might have a broader spectrum of interaction and activities than does VEGF₁₂₁, for example, with non-endothelial cells.

A recent report has demonstrated that glioma cells express a 190 kDa protein that binds VEGF₁₆₅ efficiently but not VEGF₁₂₁ (Omura et al., 1997). No stimulation of tyrosine phosphorylation of this receptor could be demonstrated upon binding of VEGF₁₆₅. Whether the 190 kDa receptor is related to neuropilin-1 is not known presently.

In summary, we have demonstrated by independent purification and expression cloning methods that the VEGF isoform-specific receptor VEGF₁₆₅R is identical to neuropilin-1, a neuronal cell surface protein previously identified as a receptor for the collapsin/semaphorin family. Furthermore, expression of neuropilin-1 enhances the binding of VEGF₁₆₅ to KDR and VEGF₁₆₅ bioactivity. Future studies are aimed at elucidating the mechanisms by which neuropilin-1 mediates VEGF₁₆₅ bioactivity.

Experimental Procedures

Materials

Cell culture media, lipofectin, and lipofectamin were purchased from Life Technologies. Human recombinant VEGF₁₆₅ and VEGF₁₂₁ were produced in Sf-21 insect cells infected with recombinant baculovirus

vectors as previously described (Cohen et al., 1992). GST VEGF exons 7+8 fusion protein was prepared in *E. coli* and purified as previously described (Soker et al., 1996). Heparin, hygromycin B, and protease inhibitors were purchased from Sigma (St. Louis, MO). ^3H -thymidine, ^{125}I -Sodium, ^{32}P -dCTP, and GeneScreen-Plus hybridization transfer membrane were purchased from DuPont NEN (Boston, MA). Disuccinimidyl suberate (DSS) and IODO-BEADS were purchased from Pierce Chemical (Rockford, IL). Con A Sepharose was purchased from Pharmacia LKB Biotechnology (Piscataway, NJ). RNAzol-B was purchased from TEL-TEST (Friendswood, TX). Silver Stain kit and Trans-Blot PVDF membranes were purchased from Bio-Rad Laboratories (Hercules, CA). Multiple-tissue Northern blot membranes were purchased from Clontech (Palo Alto, CA). PolyAtract mRNA isolation kits were purchased from Promega (Madison, WI). DNA-labeling kits and molecular weight markers were purchased from Amersham (Arlington Heights, IL). pcDNA3.1 plasmid was purchased from Invitrogen (Carlsbad, CA), and pCPhygro plasmid, containing the CMV promoter and encoding hygromycin B phosphorylase, was kindly provided by Dr. Urban Deutsch (Max Planck Institute, Bad Nauheim, Germany). Restriction endonucleases and Ligase were purchased from New England Biolabs (Beverly, MA). NT-B2 photographic emulsion and X-ray film were purchased from the Eastman Kodak company (Rochester, NY).

Cell Culture

HUVEC were obtained from American Type Culture Collection (ATCC) (Rockville, MD) and grown on gelatin-coated dishes in M-199 medium containing 20% fetal calf serum (FCS) and glutamine, penicillin, and streptomycin (GPS). Basic fibroblast growth factor (2 ng/ml) was added to the culture medium every other day. Parental PAE cells and PAE cells expressing KDR (PAE/KDR) (Waltenberger et al., 1994) were kindly provided by Dr. Lena Claesson-Welsh and were grown in F12 medium containing 10% FCS and GPS. MDA-MB-231 cells and MDA-MB-453 cells were obtained from ATCC and grown in DMEM containing 10% FCS and GPS. The human melanoma cell lines RU-mel, EP-mel, and WK-mel were kindly provided by Dr. Randolph Byer (Boston University Medical School, Boston, MA), and grown in DMEM containing 2% FCS, 8% calf serum, and GPS. Human metastatic prostate adenocarcinoma (LNCaP) and prostate carcinoma (PC3) cells were kindly provided by Dr. Michael Freeman (Children's Hospital, Boston, MA) and grown in RPMI 1640 containing 5% FCS and GPS.

Purification and Protein Sequencing

Approximately 5×10^8 MDA-MB-231 cells grown in 150 cm dishes were washed with PBS containing 5 mM EDTA, scraped, and centrifuged for 5 min at $500 \times g$. The cell pellet was lysed with 150 ml of 20 mM HEPES (pH 8.0), 0.5% Triton X-100, and protease inhibitors including 1 mM AEBSF, 5 $\mu\text{g}/\text{ml}$ leupeptin, and 5 $\mu\text{g}/\text{ml}$ aprotinin for 30 min on ice, and the lysate was centrifuged at $30,000 \times g$ for 30 min. MnCl_2 and CaCl_2 were added to the supernatant to obtain a final concentration of 1 mM each. The lysate was absorbed onto a Con A Sepharose column (7 ml), and bound proteins were eluted with 15 ml 20 mM HEPES (pH 8.0), 0.2 M NaCl, 0.1% Triton X-100, and 1 M methyl- α -D-mannopyranoside at 0.2 ml/min. The elution was repeated twice more at 30 min intervals. The Con A column eluates were pooled and incubated for 12 hr at 4°C with 0.5 ml of VEGF₁₆₅-Sepharose beads containing about 150 μg VEGF₁₆₅. VEGF₁₆₅ was immobilized as described previously (Wilchek and Miron, 1982). The VEGF₁₆₅-Sepharose beads were washed with 50 ml of 20 mM HEPES (pH 8.0), 0.2 M NaCl, and 0.1% Triton X-100 and then with 25 ml of 20 mM HEPES (pH 8.0). The beads were boiled in SDS-PAGE buffer, and bound proteins were separated by 6% SDS-PAGE. Proteins were transferred to a TransBlot PVDF membrane using a semi-dry electric blotter (Hoefer Scientific), and the PVDF membrane was stained with 0.1% Coomassie brilliant blue in 40% methanol. The two prominent proteins in a 130–140 kDa doublet were cut out separately and N-terminally sequenced using an Applied Biosystems model 477A microsequencer, a service provided by Dr. William Lane of the Harvard Microchemistry Facility (Cambridge, MA).

Expression Cloning and DNA Sequencing

Complementary DNA (cDNA) was synthesized from 5 μg 231 cell mRNA. Double-stranded cDNA was ligated to EcoRI adaptors and

size-fractionated on a 5%–20% potassium acetate gradient. DNA fragments larger than 2 kb were ligated to an eukaryotic expression plasmid, pcDNA3.1. The plasmid library was transfected into *E. coli* to yield a primary library of approximately 1×10^9 individual clones. A portion of the transformed bacteria was divided into 240 pools, each representing approximately 3×10^3 individual clones. DNA prepared from each pool was used to transfect COS-7 cells seeded in 12-well dishes using the Lipofectin reagent according to the manufacturer's instructions. Three days after transfection, the cells were incubated on ice for 2 hr with ^{125}I -VEGF₁₆₅ (10 ng/ml) in the presence of 1 $\mu\text{g}/\text{ml}$ heparin, washed, and fixed with 4% paraformaldehyde in PBS. ^{125}I -VEGF₁₆₅ binding to individual cells was detected by overlaying the monolayers with a photographic emulsion, NT-B2, and developing the emulsion after 2 days as described (Gearing et al., 1989). Seven positive DNA pools were identified, and DNA from one of the positive pools was used to transform *E. coli*. The *E. coli* were subdivided into 50 separate pools and plated onto 50 LB ampicillin dishes, with each pool representing approximately 100 clones. DNA made from these pools was transfected into COS-7 cells, which were screened for ^{125}I -VEGF₁₆₅ binding as described above. Twenty positive pools were detected at this step, and their corresponding DNAs were used to transform *E. coli*. Each pool was plated onto separate LB ampicillin dishes, and DNA was prepared from 96 individual colonies and screened in a 96-well two-dimensional grid for ^{125}I -VEGF₁₆₅ binding to transfected COS-7 cells as described above. Seven single clones were identified as being positive at this step. The seven positive plasmid clones were amplified, and their DNA was analyzed by restriction enzyme digestion. Six clones showed an identical digestion pattern of digest, and one was different. One clone from each group was submitted for automated DNA sequencing.

Northern Analysis

Total RNA was prepared from cells in culture using RNAzol according to the manufacturer's instructions. Samples of 20 μg RNA were separated on a 1% formaldehyde-agarose gel and transferred to a GeneScreen-Plus membrane. The membrane was hybridized with a ^{32}P -labeled fragment of human *neuropilin-1* cDNA corresponding to nucleotides 63–454 in the ORF at 63°C for 18 hr. The membrane was washed and exposed to X-ray film for 18 hr. A commercially obtained multiple human adult tissue mRNA blot (Clontech, 2 $\mu\text{g}/\text{lane}$) was probed for human *neuropilin-1* in a similar manner. The multiple tissue blot was stripped by boiling in the presence of 0.5% SDS and reprobed with a ^{32}P -labeled fragment of *KDR* cDNA corresponding to nucleotides 2841–3251 of the ORF (Terman et al., 1992).

Transfection of PAE Cells

Human *neuropilin-1* cDNA was digested with XhoI and XbaI restriction enzymes and subcloned into the corresponding sites of pCPhygro to yield pCPhygro-NP-1. PAE and PAE/KDR cells were grown in 6 cm dishes and transfected with 5 μg of pCPhygro-NP-1 using Lipofectamine according to the manufacturer's instructions. Cells were allowed to grow for an additional 48 hr, and the medium was replaced with fresh medium containing 200 $\mu\text{g}/\text{ml}$ hygromycin B. After 2 weeks, isolated colonies ($5\text{--}10 \times 10^3$ cell/colony) were transferred to separate wells of a 48-well dish and grown in the presence of 200 $\mu\text{g}/\text{ml}$ hygromycin B. Stable PAE cell clones expressing *neuropilin-1* (PAE/NP-1) or coexpressing *neuropilin-1* and *KDR* (PAE/KDR/NP-1) were screened for VEGF₁₆₅ receptor expression by binding and cross-linking of ^{125}I -VEGF₁₆₅. For transient transfection, PAE/KDR cells were transfected with *neuropilin-1* as described above, and after 3 days ^{125}I -VEGF₁₆₅ cross-linking analysis was carried out.

Radioiodination of VEGF, Binding, and Chemical Cross-linking

The radioiodination of VEGF₁₆₅ and VEGF₁₂₁ using IODO-BEADS was carried out as previously described (Soker et al., 1997). The specific activity ranged from 40,000–100,000 cpm/ng protein. Binding and cross-linking experiments using ^{125}I -VEGF₁₆₅ and ^{125}I -VEGF₁₂₁ were performed as previously described in the presence of 1 $\mu\text{g}/\text{ml}$ heparin (Gitay-Goren et al., 1992; Soker et al., 1996). VEGF binding was quantitated by measuring the cell-associated radioactivity in a

γ -counter (Beckman, Gamma 5500). The counts represent the average of three wells. All experiments were repeated at least three times and similar results were obtained. The results of the binding experiments were analyzed by the method of Scatchard using the LIGAND program (Munson and Rodbard, 1980). 125 I-VEGF₁₆₅ and 125 I-VEGF₁₂₁ cross-linked complexes were resolved by 6% SDS/PAGE, and the gels were exposed to X-ray films that were subsequently scanned by using an IS-1000 digital imaging system (Alpha Innotech Corporation).

Proliferation and Chemotaxis Assays

VEGF-induced proliferation was measured by adding increasing amounts of VEGF to HUVEC in gelatin-coated 96-well dishes, and DNA synthesis was measured as described previously (Soker et al., 1997). The preparation of the GST exon 7 + 8 fusion proteins and their use in HUVEC proliferation assays have been previously described (Soker et al., 1996, 1997). VEGF-induced chemotaxis was measured in a Boyden chamber as described previously (Yoshida et al., 1996; Elenius et al., 1997). Briefly, PAE, PAE/NP-1, PAE/KDR, and PAE/KDR/NP-1 cells were added to wells in the upper chamber and increasing amounts of VEGF isoforms were added to wells in the lower chamber. After a 4 hr incubation, the number of cells migrating through the filter were counted. A checkerboard analysis was used to ascertain that the migration was due to chemotaxis.

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GenBank Accession Numbers

Accession numbers for human *neuropilin-1* and human *neuropilin-2* are AF016050 and AF016098, respectively.